Attorney's Docket No. 33339/206076

PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re:	Laurent Benabis *et al.*	Confirmation No.:	
Appl. No.:	09/700,687	Group Art Unit:	1651
Filed:	February 14, 2001	Examiner:	R. Davis
For:	MUTANT LACTOBACILLUS BULGARICUS STRAINS FREE FROM BETA-GALACTOSIDASE ACTIVITY		

#13
2/12/03

January 14th, 2003

Commissioner for Patents
Washington, DC 20231

DECLARATION UNDER 37 C.F.R. 1.132

Sir:

I, J. Mengaud, do hereby declare and say as follows:

1.

CURRICULUM VITAE

Jérôme MENGAUD
Birth date : September 4th 1965
French Nationality

Professional address : DANONE Vitapole, route départementale 128, 91767 Palaiseau Cedex.
Tel : 33 (0) 1 69 35 72 98
Fax : 33 (0) 1 69 35 76 94
Email : jmengaud@danone.com

Current professional activity (since 2001) : Research group leader – Culture and Yeast. This group is part of Vitavaleur supervised by F. Gendre

Previous professional activity :

1996 - 2001 : Research engineer in the Starter an Yeast group of the Danone Research center
1993 - 1996 : Research assistant at the Pasteur Institute in Paris, Bacteria-Host interaction unit (P. Cossart)
1992 - 1993 : Post-doctoral position at the University of California Los Angeles (M. Horwitz's laboratory)

Education :
- pHD thesis in Microbiology prepared at the « Institut National d'Agronomie – Paris-Grignon » and the Pasteur Institute - Paris (December 1990) Under the Scientific supervision of P. Cossart :
Genetic analysis of *L. monocytogenes* virulence factors : role of listeriolysin O and other virulence factors.

- Engineer diploma of the « Institut Agronomique Paris-Grignon » (1987)

- DEA of microbiology of the university Paris VII (1987)

2. Since 1996, I have been working under the supervision of L. Benbadis and then F. Gendre.

3. I conducted and supervised experiments to determine whether the acidification properties exhibited by the *L. bulgaricus* lacZ$^-$ non-sense mutant of the above-referenced patent application are different from the acidification properties exhibited by a lacZ$^-$ deletion mutant derivative of this same strain of *L. bulgaricus.*

4. The experiments were conducted in the following manner.

5. In order to compare the acidification properties of the lacZ$^-$ non-sense mutant of the above-referenced patent application with a lacZ$^-$ deletion mutant in the same genetic background, the following strains of *L. bulgaricus* were used:

a) Wild-type strain LbS, which contains a full length, wild type *lacZ* gene.

b) Deletion mutant strain DN-100 542, which is derived from strain LbS and has a deletion mutation in the *lacZ* gene. Deletion mutant strains I-1067 and I-1068 (described in U.S. Patent No. 5,382,438) were also included in order to compare the deletion mutation in the *lacZ* gene of strain DN-100 542 to the deletion mutations in the *lacZ* gene of these strains.

c) Non-sense mutant strain I-1968, the strain of the above-referenced patent application, which is derived from strain LbS and has a non-sense mutation in the *lacZ* gene.

6. To confirm the genetic characteristics of the *lacZ* gene of strains DN-100 542 and I-1968, PCR amplification and sequencing methods were used to analyze the *lacZ* gene in each of these strains (see Figures 1 and 2 for experimental data; see Figure 3 for a summary of the results). PCR amplification of the *lacZ* region was accomplished by extraction of total DNA followed by amplification using two oligonucleotide primers located at the 5' (primer OFF505, corresponding to positions 1855-1879 of SEQ ID NO:1 of WO99/61627; T_d=66°C) and 3' (primer OFF527, corresponding to positions 4931-4907 of SEQ ID NO:1 of WO99/61627; T_d=66°C) ends of the *lacZ* gene. As a control for DNA quality, total DNA was separately amplified with two oligonucleotides corresponding to the 5' (primer OFF216; T_d=64°C) and 3' (primer OFF217; T_d=58°C) ends of an internal fragment of *epsA*, a gene conserved among all *S. thermophilus* strains. The sequences of these four primers are given in Table 1. PCR reaction conditions for amplification of the *lacZ* and *epsA* fragments were as follows: 94°C, 5 min; 94°C, 30 sec; 62°C (for *lacZ*) or 60°C (for *epsA*), 30 sec, 35 cycles; 72°C, 3 min 10 sec (for *lacZ*) or 1 min (for *epsA*); 72°C, 15 min. Amplified fragments were analyzed by electrophoresis on a 1% agarose gel and stained using ethidium bromide.

Table 1: PCR Primer Sequences

Primer	Sequence (5' to 3')
OFF505	GTTAGAAGGGAAGAATTAGAAAATG
OFF527	TTTCATCCTGTTAAGTCAATTGTAG
OFF216	CCGCCGTAAGCGTCATATCG
OFF217	CAAGAGGACTGGCTTCTTG

As shown in Figure 1 (lanes 2-6), all DNA preparations allowed amplification of a 210 bp fragment internal to the *epsA* gene using primers OFF216 and OFF217, confirming the quality of the DNA used in the experiments. Using the same DNA preparations and PCR reaction mixtures, the following results were obtained for the *lacZ* gene using primers OFF505 and OFF527 (see Figure 2):

a) Wild type strain LbS allowed amplification of a *lacZ* gene fragment corresponding to the 3077 bp fragment predicted from the locations of the primers on the *lacZ* gene (lane 3).

b) Deletion mutant strain DN-100 542 did not allow amplification of a *lacZ* gene fragment, indicating that either one or both of the primer regions are deleted from the *lacZ* gene region of this strain (lane 6). This result is similar to that obtained for deletion mutant strain I-1067 (lane 5), which also has a large deletion in the *lacZ* gene region (see U.S. Patent No. 5,382,438). In contrast, deletion mutant strain I-1068 allows amplification of a *lacZ* fragment slightly shorter than that of the wild type sequence (lane 2 versus lane 3), indicating a deletion of about 40 bp located somewhere between the two primers OFF505 and OFF527. This is in agreement with the description of strain I-1068 in U.S. Patent No. 5,382,438

c) Non-sense mutant strain I-1968 of the above-referenced patent application allows amplification of a fragment of the same size as that obtained for wild type strain LbS (lane 4 versus lane 3), indicating that there are no large-scale changes in the *lacZ* gene of this strain. Sequencing of this fragment revealed that the only difference between the *lacZ* gene of strain I-1968 and that of the wild type strain LbS is the presence of a non-sense mutation in the *lacZ* gene of strain I-1968 at a position corresponding to position 4519 in SEQ ID NO:1 of patent WO99/61627.

Thus, as summarized in Figure 3, these results confirm that lacZ$^-$ strains DN-100 542 and I-1968 are of a different nature: like strains I-1067 and I-1068, strain DN-100 542 has a deletion in its *lacZ* gene; in contrast, strain I-1968 does not contain a deletion mutation in the *lacZ* region but instead harbors a non-sense mutation in position 4519 of that gene (SEQ ID NO:1 of patent WO99/61627).

7. Because deletion mutant strain DN-100 542 and non-sense mutant strain I-1968 occur in the same genetic background (i.e., *L. bulgaricus* strain LbS), the acidification properties of these two strains may be directly compared. Thus a first subculture (inoculated with 1% of an overnight culture) was prepared at 44°C in sterilized reconstituted skim milk complemented with 2g/l of yeast extract and 20g/l of glucose and used to prepare a second subculture under the same experimental conditions (inoculation at 1%). Reconstituted skim milk (12% w/vol) complemented with 7g/l of glucose was heated 30 min at 95°C, inoculated with 1% of this second subculture, and the kinetics of acidification determined by measuring the pH of the resulting solution every minute at 44°C using a cinac apparatus. The experiment was done in duplicate for each strain.

As the acidification data of Figure 4 show, although deletion mutant strain DN-100 542 and non-sense mutant strain I-1968 are both lacZ$^-$, the nature of the lacZ$^-$ mutation of each results in very different acidification properties for the strains, and therefore produce very different technological properties. Specifically, strain I-1968 acidifies milk slower than strain DN-100 542. This result presents a potentially important technological property for yogurt production, since slow acidification can give more time for an associated *S. thermophilus* strain to grow, a result that is expected to be of particular importance when the *S. thermophilus* strain associated with the L. *bulgaricus* strain is a texturing strain (better growth of the *S. thermophilus* strain leads to a better texture of the end product).

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

J. Mengaud

Date 14.01.03